

Selective extraction of cotton fiber cytoplasts to identify cytoskeletal-associated proteins[§]

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(Received February 2, 1999; accepted November 10, 1999)

Abstract — Cytoplasts from cotton (*Gossypium hirsutum* L.) fiber cells retain microtubule and microfilament cytoskeletons through extraction with non-ionic detergent and ethylene glycol bis-(β -aminoethyl ether) N,N,N',N'-tetraacetic acid. Tubulin and actin are the most abundant proteins in extracted cytoplasts; however, many other less abundant proteins are also present. To determine if minor proteins were associated with the cytoskeleton, microtubules and microfilaments were selectively removed from extracted cytoplasts by detergent extraction in an alkaline Ca^{2+} solution. Under these extraction conditions, microtubules and microfilaments were fragmented and depolymerized unless previously stabilized by taxol and phalloidin. Associated proteins were identified by their loss in conjunction with either microtubules or microfilaments. As judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, one protein, of roughly 115 kDa, appeared to be associated with microfilaments since it was present in Ca^{2+} -extracted preparations only when microfilaments were stabilized with phalloidin. The failure of most minor proteins to associate with microtubules and microfilaments suggests that caution must be used when interpreting co-isolation as evidence for an association of low abundance proteins with cytoskeletons. USDA-ARS © 2000 Published by Éditions scientifiques et médicales Elsevier SAS

Actin / cytoskeleton / microfilaments / microtubules / tubulin

DAPI, 4,6-diamidino-2-phenylindole / DIC, differential interference contrast / EGTA, ethylene glycol bis-(β -aminoethyl ether) N,N,N',N'-tetraacetic acid / IF, intermediate filaments / MFs, microfilaments / MTs, microtubules / PIPES, piperazine-N,N'-bis[2-ethanesulfonic acid] / PMSE, phenylmethylsulfonyl fluoride / TRIS, 2-amino-2-(hydroxymethyl)-1,3-propanediol

1. INTRODUCTION

Arrays of MTs and MFs serve a variety of functions in plant cells [12, 25], but MT- and MF-associated proteins that presumably assist in carrying out those functions are just beginning to be characterized [7, 9, 19, 22, 24, 26]. Spectrin and IF networks may also be present, but evidence for their existence remains tentative [3, 11, 18, 24]. Given this uncertainty, insights into the composition of the plant cytoskeleton may be provided by investigating the proteins in plant cells after detergent extraction. We have developed a pro-

cedure for extracting cotton (*Gossypium hirsutum*) cytoplasts with non-ionic detergent [3]. By analysis with SDS-PAGE, these preparations were found to contain a few abundant proteins and many minor proteins. The abundant proteins proved to be cytoskeletal proteins, actin and tubulin, but the identity of the minor proteins, whether cytoskeletal proteins or contaminants, could not be determined.

We sought a method for judging if any of the minor proteins in extracted cytoplasts was associated with the plant cytoskeleton. One approach is to selectively remove one cytoskeletal array (e.g. MFs) from a sample and monitor the concomitant loss of other proteins. If the method for removing the cytoskeletal array does not affect any other protein interactions, only the components of that array plus its associated proteins should be lost from the sample. Comparison of samples with and without a given cytoskeletal array

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would permit the identification of proteins directly and specifically associated with that array.

The selective removal of MTs and MFs should be possible by extracting cytoplasts under conditions that depolymerize MTs and MFs unless specifically stabilized by taxol and phalloidin, respectively. In the absence of taxol and phalloidin, alkaline Ca^{2+} solutions are suitable for fragmenting/depolymerizing both MTs [5, 27] and MFs [2, 15]. Taxol, an alkaloid, binds to MTs and prevents their depolymerization [5, 6, 21]. Similarly, the cyclic peptide phalloidin binds to and stabilizes MFs [1, 23].

We have developed a method using Ca^{2+} , taxol and phalloidin to selectively remove MTs or MFs during the detergent extraction of cotton fiber cytoplasts. By comparing the protein compositions of cytoplasts with MTs or MFs removed, we were able to judge if any of the minor proteins present in cytoplast preparations were specifically associated with MT or MF arrays.

2. RESULTS

Cytoplasts derived from cotton fiber cells contained a large central vacuole intersected by cytoplasmic strands and surrounded by a cortical cytoplasm. After extraction with Triton X-100 at pH 6.8 in the absence of Ca^{2+} , a fibrous 'ghost' was formed in the shape of the original cytoplast. Examples of extracted cytoplasts are shown in *figure 1 A*. As described previously [3], these cytoplasts retained MF and MT arrays reminiscent of those within intact fiber cells.

When extracted in an alkaline Triton solution containing Ca^{2+} , cytoplasts became very fragile. On isolation, the extracted cytoplasts collapsed into dense aggregates around nuclei or wall fragments, as shown in *figure 1 B*. The inclusion of taxol and phalloidin during the detergent extraction did not affect the general appearance of the protein aggregates as observed by DIC microscopy but noticeably increased the quantity.

2.1. Taxol and phalloidin selectively prevent the loss of microtubules and microfilaments

Fluorescence microscopy was used to judge if MTs and MFs were retained in the Ca^{2+} -extracted cytoplasts. Both rhodamine phalloidin and FITC-anti-mouse/anti-tubulin antibodies labeled the aggregates, especially when taxol and phalloidin were present during the extractions. An inability to clearly distinguish individual fluorescent filamentous structures in

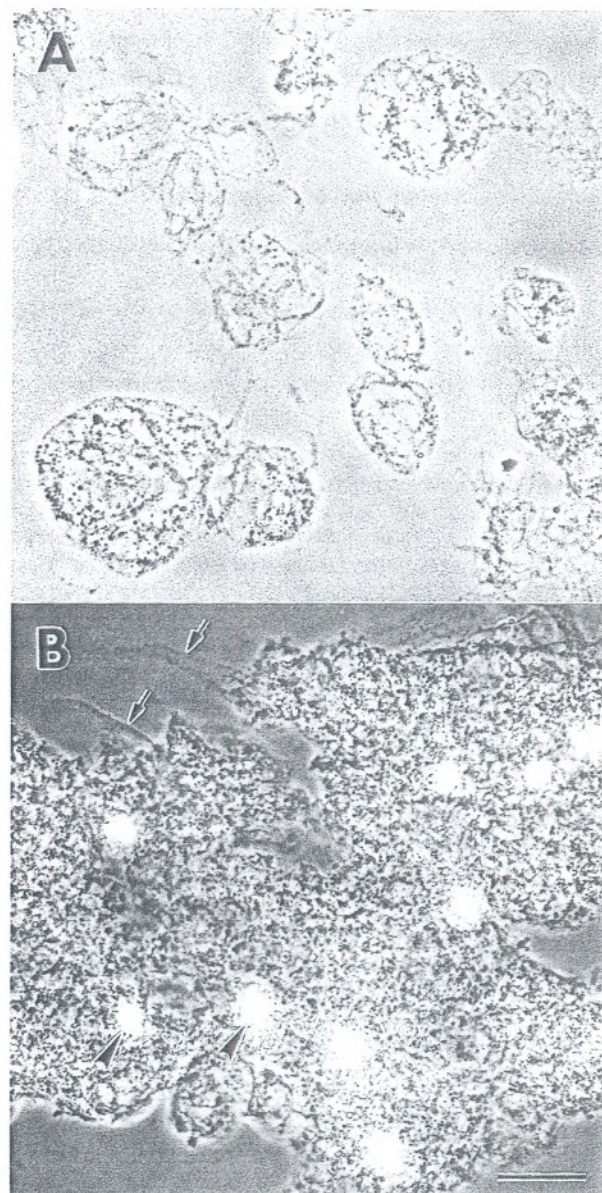


Figure 1. Fiber cell cytoplasts after extraction with Triton X-100 in the presence and absence of free Ca^{2+} . **A**, Phase contrast view of cytoplasts after extraction and isolation at pH 6.8 with Ca^{2+} chelated by EGTA. Note that the general form of the unextracted cytoplasts is retained. **B**, Phase contrast plus DAPI fluorescence view of cytoplasts after extraction at pH 8, 0.012 M free Ca^{2+} , 30 μM phalloidin and 20 μM taxol. Extracted cytoplasts were isolated into a solution including DAPI and EGTA (pH 6.8). Cytoplasts have collapsed into dense aggregates around wall fragments (arrows) and DAPI-stained nuclei (arrowheads). The scale bar represents 25 μm in both images.

the aggregates made it difficult to judge if the labeling was specific or if the MTs and MFs were actually

intact. Their condition was inferred, however, from the condition of MTs and MFs within partially digested fiber cells that contaminated the preparations. *Figure 2 A and B* show a fiber cell fragment that was detergent extracted with Ca^{2+} plus taxol. MFs were absent (*figure 2 A*), while MTs were visible in their expected transverse orientation (*figure 2 B*). *Figure 2 C and D* show a fiber cell fragment that was detergent extracted with Ca^{2+} plus phalloidin. With this treatment, MFs were retained, paralleling the fiber cell axis (*figure 2 C*), but MTs were absent (*figure 2 D*). The condition of the cytoskeletal elements in fiber cell fragments suggests that taxol and phalloidin were able to preserve MTs and MFs, respectively, in Ca^{2+} -Triton extracted cytoplasm preparations.

2.2. Taxol and phalloidin selectively prevent the loss of actin and tubulin

One-dimensional SDS gel electrophoresis and immunoblotting were used to judge qualitatively the amounts of actin and tubulin in extracted cytoplasm preparations. As expected, actin and tubulin were found in preparations that retained MFs and MTs. As shown in the immunoblot in *figure 3 A*, actin was retained in Ca^{2+} /Triton-extracted cytoplasm preparations only when phalloidin was present during the extractions (lanes 2 and 4). Similarly, tubulin was retained only when taxol was present (lanes 3 and 4).

2.3. Loss of actin or tubulin does not cause the loss of minor proteins

The profile of total proteins from extracted cytoplasm preparations were observed after SDS-PAGE and Coomassie Blue staining. The loss or retention of bands corresponding to actin and tubulin are clearly visible in *figure 3 B*, lanes 1–4. As long as actin and/or tubulin were present, the patterns of less abundant proteins were almost identical to each other (*figure 3 B*, lanes 2–4). The pattern of less abundant proteins was also nearly identical to proteins in cytoplasm extracted in the absence of Ca^{2+} (*figure 3 B*, lane 0), although the total amount of protein isolated was reduced by half in the presence of Ca^{2+} (*figure 3 B*, compare lane 0 with 4, noting that half as much protein was loaded in lane 0). In the absence of both actin and tubulin, the minor proteins were significantly reduced (*figure 3 B*, lane 1).

One minor protein was an exception; it was observed only when actin was retained during extractions (*figure 3 B* arrows, lanes 2 and 4). The molecular mass of this protein was estimated to be ~115 kDa when electrophoresed on a 6 % polyacrylamide gel.

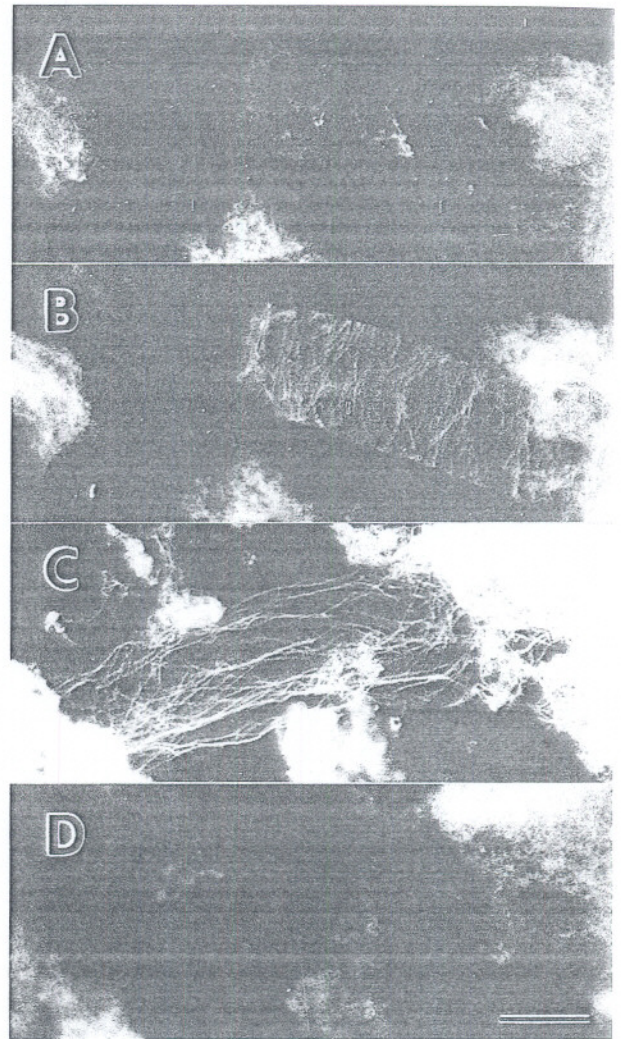


Figure 2. Double immunofluorescent staining of MFs and MTs in Ca^{2+} -Triton extracted fiber cell fragments and cytoplasm. **A**, Fiber cell fragment extracted in the presence of taxol but not phalloidin and labeled with rhodamine phalloidin. MFs are not visible. **B**, The same fragment showing anti- β -tubulin labeling. The MTs are retained and run perpendicular to the long axis of the fiber cell. **C**, Fiber cell fragment extracted in the presence of phalloidin but not taxol and labeled with rhodamine phalloidin. MFs are visible running parallel to the long axis. **D**, The same fragment as **C** showing anti- β -tubulin labeling. MTs are not visible. The scale bar represents 25 μm in all images.

Delayed phalloidin addition demonstrated that the ~115-kDa protein was not an artifact of the phalloidin addition itself, as shown by lanes 5 and 6 of *figure 3 B*. If the protein were a contaminant of the phalloidin, it would have been present in lane 6. Similarly, if phalloidin somehow induced the ~115-kDa protein to

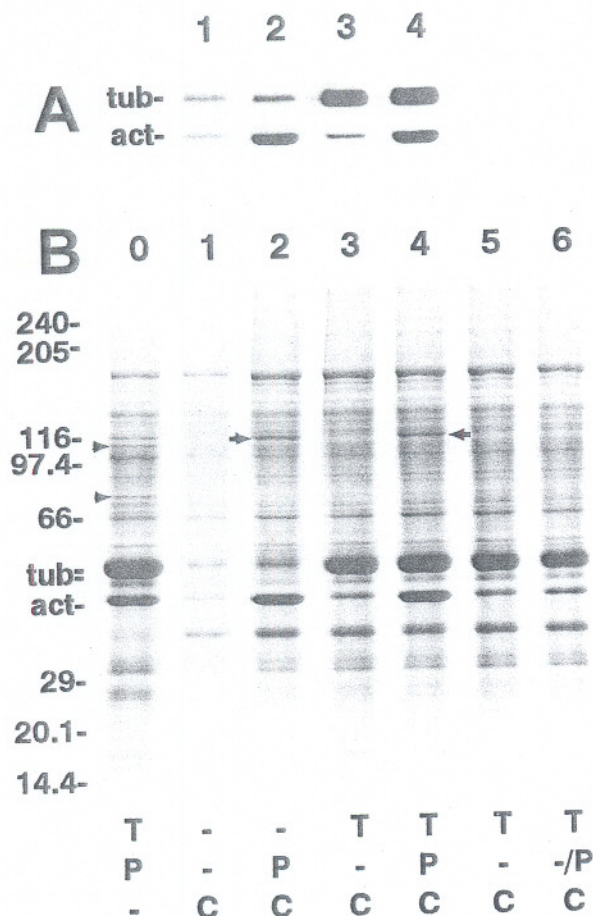


Figure 3. Immunoblot and Coomassie Blue stained gel of cytoplasmic proteins after extraction with Triton and combinations of Ca^{2+} , phalloidin and taxol. As indicated, cytoplasts were Triton-extracted in the presence of 20 μM taxol (T), 30 μM phalloidin (P), and/or 0.012 M Ca^{2+} plus 0.05 M Tris-HCl (pH 8.0) (C). Except for lane 0, equal volumes of cytoplasts were extracted, isolated and loaded on gels so that extraction conditions causing the loss or retention of specific proteins could be identified. The corresponding lanes in the gel and blot were loaded with equal sample amounts. The mobilities of molecular mass standards, actin (act) and tubulin (tub) are indicated. A, Immunoblot probed with anti-actin and anti- β -tubulin antibodies; B, Coomassie Blue stained gel. Arrows indicate the location of the ~115-kDa protein. Arrowheads by lane 0 indicate more obvious proteins lost from preparations extracted in the presence of free Ca^{2+} . Lane 0, preparation extracted 30 min; sample volume loaded is half that of other lanes. Lanes 1–4, preparations extracted 30 min. Lanes 5–6, preparations extracted 20 min, then phalloidin was added to a final concentration of 30 μM , and both samples extracted an additional 20 min.

co-isolate with the extracted cytoplasts without binding MFs, it should also have been observed in lane 6.

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3. DISCUSSION

Proteins that may associate with the cytoskeleton in higher plants have been identified by their affinity for MTs and MFs in vitro. They have been identified by their ability to re-bind to cytoskeletal polymers either through cycles of polymerization [8, 26] or in affinity chromatography [9]. Alternatively, cytoskeletal-associated proteins have been demonstrated by the ability of proteins to remain bound to cytoskeletal polymers through detergent extractions [7, 28].

The successful identification of cytoskeletal-associated proteins by either affinity-based approach requires demonstrating that the candidate proteins are actually binding MTs or MFs, first in vitro in the identification procedure, and then in vivo. Demonstrating an association in vivo is more difficult than showing an association in vitro [10, 19]. In this study, we have sought to determine in vitro the MT or MF associations of proteins in detergent-extracted cytoplasts. Phalloidin, taxol, and Ca^{2+} were used to selectively remove MTs and MFs, permitting the identification of proteins lost in conjunction with tubulin and actin.

3.1. One protein associates specifically with MFs

Under the extraction conditions used, one protein of roughly ~115 kDa appeared to associate specifically with MFs, since this protein was retained only in preparations where actin was retained. The simplest explanation for this result is that the ~115-kDa protein was associated with MFs in vivo, and remained associated through the detergent extraction. Of course, a specific association in vitro does not guarantee an association in vivo. Given that actin filaments can bind a variety of basic proteins [14], the ~115-kDa protein could be compartmentalized in vivo, and bind MFs only when freed from its compartment during detergent extraction.

Other proteins may associate with MTs or MFs, but they were not detected by the methods used here. If relatively small numbers of associated proteins are sufficient to maintain cytoskeletal structures, some associated proteins may simply have been below the detection level of the Coomassie Blue stain, or hidden within actin or tubulin bands. Alternatively, associated proteins may dissociate from MTs or MFs in alkaline Ca^{2+} solutions and be lost from the preparations during the detergent extraction. Some proteins were clearly lost from extracted cytoplasts as a result of Ca^{2+} extraction (figure 3 B, lane 0, arrowheads). Whether these proteins were associated with MFs or MTs is

unknown, but the loss of associated proteins conceivably could have contributed to the collapse of cytoplasts extracted with Ca^{2+} (figure 1 B).

3.2. Most proteins do not associate with MTs or MFs

Although most minor proteins do not specifically associate with MTs or MFs, the overall quantity of minor proteins varies with the amounts of MTs or MFs. When both cytoskeletal arrays were removed, the quantity of minor proteins decreased substantially (figure 3 B, lane 1), and when the amount of actin and tubulin were increased two-fold in the absence of Ca^{2+} , the minor proteins increased in a similar manner (compare figure 3 B, lane 0 with lane 4, noting that half as much protein was loaded in lane 0).

One possible explanation for this general association is that many of the minor proteins are contributed by a few nuclei that might contaminate the preparations. Nuclei could contribute a large number of basic proteins including histones and nuclear envelope proteins. In addition, the number of nuclei varied with the overall quantity of MTs and MFs; free nuclei generally pelleted through the 50 % sucrose while nuclei entangled in enough MTs and MFs (as in figure 1 B) were retained with the extracted cytoplasts. Although it is feasible to show that a known protein is not associated with nuclei contaminating preparations [28], it is more difficult to show that all unidentified minor proteins were associated with nuclei. With or without Ca^{2+} during extraction, nuclei co-pelleted with significant amounts of fibrous material as judged by DIC microscopy. The purity of these nuclei was insufficient to determine conclusively if nuclear proteins were the source of minor proteins in extracted cytoplast preparations.

3.3. Implications for the identification of cytoskeleton-associated proteins

It is highly likely that any preparation of isolated cytoskeletal material from plant cells will have some level of contamination by non-cytoskeletal proteins. When the level of contamination is low, all the proteins in the preparation can be assumed to have some cytoskeletal association. When the amounts of cytoskeletal or associated proteins are low, it is critical to find additional means of demonstrating that a given protein is a cytoskeletal component. Preparations of extracted cotton fiber cytoplasts appear to fall in this latter category. From this work, we can neither be certain that most of the minor proteins are associated

with the cytoskeleton, nor assume that minor proteins are non-specific contaminating proteins. This is not to imply that detergent-extracted cytoplasts, or extracted plant cells in general, are not useful for the study of cytoskeletal proteins. Although extracted cytoplasts should not be regarded as pure cytoskeletons, they are enriched in cytoskeletal components. We have exploited this enrichment to aid in the identification of a potential MF binding protein by selective extraction of MFs. Additional characterization of this protein by microsequencing will follow. In conjunction with this and other methods of identifying cytoskeletal proteins, extracted plant cell preparations can be exploited to characterize components of the plant cytoskeleton.

4. METHODS

4.1. Materials

Unfertilized ovules were harvested from greenhouse or field-grown *Gossypium hirsutum* L. plants on the day of anthesis, and used to initiate ovule cultures [4]. All chemicals were purchased from Sigma Chemical Company (St Louis, MO) unless otherwise noted. The osmolalities of solutions were determined with a Wescor Inc., model 5500 vapor pressure osmometer (Logan, UT).

4.2. Buffers

MTS buffer, 0.005 M PIPES-KOH (pH 6.8), 0.001 M EGTA, 0.005 M MgCl_2 ; Overlay buffer, 0.5 M sorbitol in MTS buffer adjusted to an osmolality of 725 mOs with glycine; Ca^{2+} -Tris buffer, 0.1 M CaCl_2 , 0.2 M Tris-HCl (pH 8.1).

4.3. Cytoplast production and extraction

Cytoplasts were produced from cotton fiber cells as previously described [3]. Cytoplasts from 16 g fresh weight of ovules were diluted to 9 mL with Overlay buffer, and the protease inhibitors aprotinin, leupeptin and pepstatin were added to give final concentrations of $5 \mu\text{g}\cdot\text{mL}^{-1}$. The cytoplasts were transferred to 2-mL microfuge tubes, 1.13 mL per tube. Phalloidin and taxol stock solutions, when used, were added to final concentrations of 30 and 20 μM respectively in a final volume of 1.5 mL. Additional protease inhibitors were added (8 μL 0.2 M PMSF, 0.1 M benzamide and 0.1 M benzamidine in ethanol) immediately followed by 75 μL 40 % v/v Triton X-100 in Overlay buffer. The tubes were capped and gently mixed by inversion.

After 5 min, 0.3 mL Ca^{2+} -Tris buffer was added, and the solutions mixed. After 20 min, each solution was layered over 0.5 mL 50 % sucrose and 1.2 mL 20 % sucrose in MTS buffer in 10×75-mm glass test tubes. After 30 min of extraction, the tubes were centrifuged 15 min at $3\,000 \times g$ in a swinging bucket rotor. The detergent solutions were aspirated, and the extracted cytoplasts were collected at the 20/50 % sucrose interface. The extracted cytoplasts from each tube were concentrated for electrophoresis by diluting to 1 mL in MTS buffer, centrifuging at $2\,500 \times g$ for 5 min, and discarding the supernatant liquid.

4.4. Electrophoresis and immunoblotting

Discontinuous SDS polyacrylamide gel electrophoresis was performed by the method of Laemmli [16] using 6–15 % acrylamide gradient gels. Gels were stained with Coomassie Blue [20]. Molecular mass standards were purchased from Pharmacia Biotech Inc. (Piscataway, NJ). Gels were blotted and probed with antibodies as described previously [3], using as primary antibodies a 1/2 600 dilution of DM1B anti- β -tubulin (Amersham Life Science Inc., Arlington Heights, IL) and a JLA20 anti-actin (NIH Developmental Studies Lab, Iowa City, IA) used at $1.5 \mu\text{g}\cdot\text{mL}^{-1}$. The secondary antibody was conjugated to alkaline phosphatase and used at 1/500 dilution. Blots were developed in nitro blue tetrazolium and bromochloroindolyl phosphate [17].

4.5. Immunofluorescence microscopy

Nuclei were stained in unfixed preparations by the addition of DAPI to 0.0005 % [13]. MTs and MFs were detected as described previously [3] by fixing with glutaraldehyde and paraformaldehyde, then incubating with DM1B anti- β -tubulin, fluorescein isodithiocyanate-conjugated anti-mouse antibody and rhodamine phalloidin.

Acknowledgments

The JLA20 anti-actin antibody was obtained from the Developmental Studies Hybridoma Bank maintained by the Department of Pharmacology and Molecular Sciences at Johns Hopkins University School of Medicine, Baltimore, MD and the Department of Biology at the University of Iowa, Iowa City, IA, under contract number NO1-HD-2-3144 from the NICHD.

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